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HRAD1 and *MRAD1* encode mammalian homologues of the fission yeast *rad1⁺* cell cycle checkpoint control gene

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ABSTRACT

Eukaryotic cells arrest at the G_2 checkpoint in the presence of DNA damage or incompletely replicated DNA. This cell cycle checkpoint prevents the development and propagation of genomic instability. In the fission yeast, this process requires the action of a number of genes, including *rad1⁺*. We report here the identification of human and mouse cDNAs that exhibit extensive sequence homology to *rad1⁺*. The human gene, called *HRAD1*, encodes a 282 amino acid protein that is 27% identical and 53% similar to yeast Rad1p. The human homologue maintains its sequence similarity over the full length of the protein, including the three proposed 3′→5′ exonuclease domains, and the leucine rich repeat region. The mouse gene, called *MRAD1*, encodes a 280 amino acid protein that is 90% identical and 96% similar to *HRAD1* at the amino acid level. Expression of *HRAD1* in yeast *rad1* mutants partially restores radiation resistance and G_2 checkpoint proficiency to these mutants. Evolutionary conservation of structure between *HRAD1*, *MRAD1*, *rad1⁺*, *Saccharomyces cerevisiae RAD17* and the *Ustilago maydis REC1* checkpoint genes suggests that the function of the encoded proteins is conserved as well. The ability of *HRAD1* to partially complement yeast *rad1* mutants suggests that this gene is required for G_2 checkpoint control in human cells.

INTRODUCTION

Cell cycle checkpoints are regulatory mechanisms that ensure prerequisite events are completed before subsequent cell cycle transitions occur. For example, mitotic entry is dependent on the prior completion of DNA replication. Checkpoints also exist to prevent the propagation of damaged chromosomes that can result from radiation or radiomimetic drugs. These DNA damage checkpoints operate predominantly at the G_1/S and G_2/M transition points (1).

Even in the absence of exogenous DNA damage or blocked DNA replication, checkpoint mutants are known to exhibit genomic instability, as seen in *RAD9* mutants of *Saccharomyces cerevisiae* at the G_2/M checkpoint (2), and in *p53*$^{-/-}$ mammalian cell lines at the G_1/S checkpoint (3,4). The accumulation of mutations in cells exhibiting genomic instability has been suggested to be the driving force behind tumour formation and metastasis (5,6). This is supported by studies on individuals with inherited chromosome instability diseases which include ataxia telangiectasia , Li-Fraumeni syndrome and Bloom's syndrome (7–10). In all three cases, genomic instability and cancer predisposition are seen, with the former operating at the cellular level and the latter at the level of the individual. The genes mutated in these diseases are *ATM*, *p53* and *BLM*, respectively. The *ATM* protein is a member of the PI-3 kinase family (11,12) and p53 is a transcription factor (13–15), and both are known to have checkpoint functions (16–23). The *BLM* protein has structural homology with known helicases and is also thought to function in checkpoint control (24,25).

A recent report has shown a strong correlation between loss of the G_2 checkpoint and the appearance of chromosomal abnormalities (26), suggesting that the G_2 checkpoint is a major protective factor against the development of genomic instability and cancer. Despite its apparent importance, only two presumptive components of the mammalian G_2 checkpoint have been identified to date (27–29). By contrast, the G_2 checkpoint has been well characterised in the fission yeast *Schizosaccharomyces pombe*. Fission yeast undergo a dose dependent G_2 delay following exposure to radiation and the resultant DNA damage that occurs (30,31). The yeast remain arrested at G_2 while the damage is repaired, then enter mitosis and resume progression through the cell cycle. This dose dependent response to radiation is absent from mutants of any one of the six checkpoint *rad* genes *rad1*$^+$, *rad3*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$ and *hus1*$^+$ (30–33). Mutants of any one of these genes have similar phenotypes; they are hypersensitive to radiation and to transiently inhibited DNA replication, such as occurs in the presence of hydroxyurea (HU). The sensitivity of these mutants to radiation and HU results from loss of the G_2 DNA damage checkpoint and the S phase checkpoint monitoring completion of DNA synthesis, respectively (30–33).

The fission yeast *rad1*$^+$ gene has previously been shown to be conserved among lower eukaryotes. *Saccharomyces cerevisiae*

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RAD17 (34) and *Ustilago maydis REC1* (35,36) are functional homologues of *rad1*$^+$. *RAD17* and *REC1* were shown independently to be required for checkpoint function, and both exhibit moderate sequence conservation with *rad1*$^+$ (25–30% at the amino acid level). We report here the cloning of human and mouse homologues of the *S.pombe rad1*$^+$ gene, called *HRAD1* and *MRAD1*, respectively. Expression of *HRAD1* in yeast *rad1* mutants results in partial restoration of the G_2 checkpoint response to radiation. Expression of *HRAD1* in these yeast does not restore resistance to HU. We propose that *HRAD1* and *MRAD1* are components of the G_2 checkpoint mechanism in humans and mice, respectively.

MATERIALS AND METHODS

DDBJ/EMBL/GenBank accession numbers

The accession numbers for the *HRAD1* and *MRAD1* cDNA sequences are AF011905 and AF038841, respectively.

cDNA libraries, screening and sequencing

The HaCaT cDNA library in λ ZAP II was a gift of D.Beach, and the CB7 mouse erythroleukemia cDNA library was a gift from P.A.Greer. A probe for screening the HaCaT cDNA library was generated by amplification of a 399 bp portion of the EST sequence (DDBJ/EMBL/GenBank accession no. AA029300) using primers A (GGTACATGACCTTGCTCCTAT) and B (AGTTCCCACCTTGACTATCC), and HaCaT cDNA as template. The full-length *HRAD1* cDNA was used as a probe to screen the mouse cDNA library. Library screens were performed using standard techniques (37). Sequencing of both strands of the *HRAD1* and *MRAD1* cDNAs was performed on an ABI 377 automated sequencer after subcloning into pBluescript KS$^-$. Amino acid sequence alignments were generated using the CLUSTAL W program (38). In the amino acid alignment, similar amino acids are defined as I/L/V/M, D/E, S/T, A/G, N/Q, R/H/K and W/F/Y.

Plasmid constructions

The *rad1*$^+$ cDNA was excised from pGR4-rad1$^+$ (gift of S.E.Sorensen) with *Bam*HI and *Xba*I, the 3′ recessed ends were filled in with Klenow polymerase, and the cDNA was blunt end ligated into the *Sma*I site of the *S.pombe* expression vector pART1 (39), to generate pART1-rad1$^+$. The *HRAD1* open reading frame (ORF) was amplified with primers HRAD1-5 (GGACGGTCGACATGCCCCTTCTGACCCAA) and HRAD1-3 (ACGGATCCTCAAGACTCAGATTCAGG), and blunt end ligated into the *Sma*I site of pART1, to generate pART1–HRAD1. Orientation of the inserts within pART1 was determined by restriction enzyme digestion.

Schizosaccharomyces pombe culture and manipulations

Schizosaccharomyces pombe was cultured using standard techniques (40). The strains used in this study were Sp337, *h*$^{+N}$ *rad1::ura4*$^+$ *leu1-32 ura4-D18*; and Sp199, *h*$^{+N}$ *cdc25-22 rad1-1 leu1-32*. Sp337 was generated by crossing 975 (40) with Sp267 (41), and Sp199 was generated by crossing SP32 (41) with SP1202 (41). *Schizosaccharomyces pombe* transformations were performed using the method of Okazaki *et al*. (42).

Radiation sensitivity and radiation-induced cell cycle delay

X-irradiation was delivered using a Clinac 2100 C/D with a 6 MV beam, at a dose rate of 0.24 Gy/s. UV radiation treatments were performed at 254 nm, with a dose rate of 1.8 J/m^2/s. For viability assays, *S.pombe* was cultured to mid-logarithmic phase (5×10^6 cells/ml) at 25°C, plated on minimal selective media at a density of 1000 cells per plate, and irradiated with the indicated dose of radiation. The plates were incubated at 30°C until colonies were easily visible. Relative viability was expressed as the number of treated versus untreated cells that were able to form colonies.

To assess radiation-induced checkpoint control, plasmids were transformed into a *cdc25-22 rad1-1* strain background. These cells were cultured to mid-logarithmic phase at 25°C, plated on pre-warmed minimal selective plates, and incubated at 36°C for 3 h to synchronize the cells in G_2. Immediately prior to release from 36°C, the plated cells were irradiated with the indicated dose of UV radiation, transferred to liquid minimal selective media, and incubated at 25°C. Samples were removed at the indicated times and fixed in 3.7% formaldehyde. Fixed cells were washed once with phosphate buffered saline (PBS), once with PBS containing 1% Triton X-100, and resuspended in PBS. The cells were then stained with 0.2 μg/ml 4′6-diamidino-2-phenylindole (DAPI) and viewed under a fluorescence microscope. Binucleate cells were scored as having passed mitosis.

Sensitivity to HU

Schizosaccharomyces pombe was cultured to mid-logarithmic phase at 32°C, and then HU was added to a final concentration of 12 mM. At the indicated times after the addition of HU aliquots of cells were removed, and plated on PM media at a density of 1000 cells per plate. The plates were incubated at 30°C until colonies had reached a suitable size for counting, and relative viability was assessed as described above for radiation sensitivity.

RESULTS

Isolation of the *HRAD1* and *MRAD1* genes

A search of the dBEST data base revealed an EST of interest obtained from a normalized and directionally cloned human cDNA library (43). The complementary strand of the EST appears to encode a predicted protein similar to the *S.pombe rad1*$^+$ gene product. This ORF predicted a protein that is 30% identical and 57% similar over an 80 amino acid stretch, which represents approximately one quarter of the Rad1p protein. It is aligned closer to the C-terminal portion of the protein which is a moderately conserved region in the *S.pombe rad1*$^+$, *S.cerevisiae RAD17* and *U.maydis REC1* gene products. The extent of homology in the region that the EST is aligned with *S.pombe rad1*$^+$ is comparable to that of *rad1*$^+$ and *RAD17* (44). This same region contains nine identical residues between Rad1p, RAD17p and REC1p, of which seven are also present in the human EST. Based on the alignment and extent of sequence identity, this was evidence for the existence of a possible human homologue of *S.pombe rad1*$^+$.

Because a positive orientation clone had not been identified in the original library, we chose to search other cDNA libraries for the *bona fide* human *rad1*$^+$ homologue. A HaCaT (spontaneously transformed human keratinocyte) cDNA library in λ ZAP II was amplified by PCR using oligonucleotide primers directed against

the putative *HRAD1* gene. The 399 bp PCR product generated using oligonucleotides A and B was subcloned into pBS KS$^-$. Sequencing of the subclone revealed an insert of identical sequence to that of the original EST, confirming that the sequence of interest was present in the HaCaT cDNA library.

The screen of the HaCaT cDNA library yielded four positive clones. *In vivo* excision converted these λ cDNA vectors into pBS plasmids containing the cDNA insert. Sequencing indicated that all four contained the same cDNA. One of these, clone HRAD1-7, was slightly longer than the others and was chosen for further analysis.

The full-length HRAD1-7 clone was used to probe a mouse CB7 erythroleukemia cDNA library by low stringency hybridization. Five positives were identified, four of which were the same length, and one was slightly shorter than the others. Clone MRAD1-2.1 was chosen for further analysis.

Sequence analyses of the *HRAD1* and *MRAD1* genes

Full DNA sequences of both strands of the insert of clone HRAD1-7 showed that the cDNA was 1300 bp long with a 214 bp 5′ untranslated region (UTR), an 846 bp coding region and a 240 bp 3′ UTR (Fig. 1A). The 3′ UTR contains a consensus AATAAA polyadenylation signal sequence. The ORF of *HRAD1* encodes a 282 amino acid polypeptide with 27% identity and 53% similarity to Rad1p. This is 41 amino acids shorter than the *S.pombe rad1*$^+$ gene product.

Complete sequencing of both strands of clone MRAD1-2.1 identified a cDNA that was 1380 bp long with a 218 bp 5′ UTR, an 840 bp coding region and a 322 bp 3′ UTR (Fig. 1B). The 3′ UTR contains a common variant of the consensus polyadenylation signal sequence (ATTAAA). However, no poly A tail is observed in this cDNA isolate. The ORF of *MRAD1* encodes a 280 amino acid polypeptide that is 90% identical and 96% similar to HRAD1p. An amino acid alignment (Fig. 2) shows that the sequence similarity of HRAD1p and MRAD1p to the other members of the Rad1p family extends over their entire lengths, suggesting that the isolated human and mouse cDNAs are full-length.

A.

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GAATTCGGCA CGAGCCGAGG TGGAGGGCCG GTCTGAAGAG TGGCGGGACT GGCTTCACTT   60
CCTCCGCGGT TCCTCGGAGC CGCCTCGCTC CTCTTCAGGG ACTTTGCTGA GAAGGGCTCT  120
CGGGCGTCCA GACCCCACCG CAAAGGTGTT TGGCGATCCG CCGAGAAGTT GTTGGCCCCA  180
GGAGCATCCC TCGGGGCCGA ATGCGCAGTG GACGATGCCC CTTCTGACCC AACAGATCCA  240
AGACGAGGAT GATCAGTACA GCCTTGTGGC CAGCCTTGAC AACGTTAGGA ATCTCTCCAC  300
TATCTTGAAA GCTATTCATT TCCGAGAACA TGCCACGTGT TTCGCAACTA AAAATGGTAT  360
CAAAGTAACA GTGGAAAATG CAAAGTGTGT GCAAGCAAAT GCTTTTATTC AGGCTGGAAT  420
ATTTCAGGAG TTTAAAGTTC AGGAAGAGTC TGTTACTTTT CGAATTAATT TAACTGTCCT  480
TTTAGACTGT TTATCTATTT TTGGATCAAG TCCTATGCCA GGGACTTTAA CTGCACTTCG  540
AATGTGTTAC CAAGGTTATG GTTACCCTTT GATGCTGTTC CTGGAAGAAG GAGGAGTGGT  600
GACAGTCTGC AAAATCAATA CACAGGAACC TGAGGAGACC CTGGACTTTG ATTTCTGCAG  660
CACCAATGTT ATTAATAAAA TTATTCTGCA GTCAGAGGGG CTCCGTGAAG CATTTTCTGA  720
ATTGGATATG ACGAGTGAAG TCCTACAAAT TACCATGTCT CCTGACAAGC CTTATTTCAG  780
GTTATCTACT TTTGGAAATG CAGGAAGTTC CCACCTTGAC TATCCCAAAG ATTCTGATTT  840
GATGGAAGCA TTTCATTGTA ATCAGACCCA AGTCAACAGA TACAAGATTT CCTTACTGAA  900
ACCCTCTACA AAGGCATTAG TCCTATCTTG TAAGGTATCT ATTCGGACAG ATAACAGAGG  960
CTTCTTTTCA TTACAGTATA TGATTAGAAA TGAAGATGGA CAAATATGTT TTGTGGAATA 1020
TTACTGCTGC CCTGATGAAG AAGTTCCTGA ATCTGAGTCT TGAGTATGAC AATTCACTGA 1080
TATTTATGTG TACATTTATG ATAGATGAAG TTCTTATTCT GAGTACAGTA CTCTTTGTCA 1140
TTTCATATTG GATTTTCTAT AGAGAAGAAG CACAATGGGG AAGATAGGAG CAAGGTCATG 1200
TACCCTAATA GTTACTATGT TTTGTAAATC CATTTTGTAG AGGGCATGTA AATAAATGTT 1260
TTCCTGTAGT CATAGATTAA AAAAAAAAAA AAAACTCGAG                        1300
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B.

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GAATTCGCGG CCGCGCTTTT TGGACGCTCA GGGTGTTCTT GGGGCTGGGG TGGCAGGGGC   60
TTTGAGGGGT CGGACGTCAC CCACAGCCTG GCGCCAGGGG CTCCCTTAGT GGCCGCTGTG  120
CGGCGAGCGG GCCGAAGTCG CGTTCTCCTG CAAGCCAGGT TTTCACACAT CTTCCGGAAG  180
AGGAGCATCC TTCTTCTCGG AGCCGACGGT ACCGTGGACC ATCCATGCCT CTCCTAACCC  240
AGTACAATGA AGAGGAGTAC GAACAGTACT GCTTAGTGGC CAGCCTTGAC AACGTTAGGA  300
ATCTCTCCAC TGTCTTGAAA GCCATTCATT TCAGAGAACA CGCCACGTGT TTTGCTACCA  360
AAAACGGAAT CAAGGTTACA GTGGAGAATG CAAAGTGTGT GCAAGCAAAT GCCTTTATTC  420
AGGCTGACGT GTTTCAGGAA TTTGTCATTC AGGAAGAATC TGTTACTTTT CGAATTAACT  480
TAACTATCCT TTTAGACTGT TTATCTATTT TTGGATCAAG TCCTACACCA GGGACTTTGA  540
CTGCGCTTCG GATGTGTTAC CAAGGTTATG GTCACCCACT GATGCTATTT CTAGAAGAAG  600
GAGGAGTGGT GACGGTCTGC AAAATTACCA CTCAGGAGCC TGAGGAGACA CTGGATTTTG  660
ATTTCTGCAG CACCAATGTT ATGAATAAAA TTATCCTGCA GTCAGAGGGG CTCCGGGAAG  720
CCTTTTCTGA GCTGGACATG ACAGGTGATG TCCTACAGAT CACTGTGTCT CCTGACAAGC  780
CCTATTTCAG GTTGTCTACT TTTGGAAATG CAGGAAACTC CCATCTTGAC TATCCCAAAG  840
ATTCCGACTT GGTGGAAGCC TTTCACTGTA ATAAGACCCA GGTCAACAGA TACAAGCTGT  900
CGCTACTGAA GCCCTCTACA AAGGCACTAG CTTTATCCTG TAAAGTGTCT ATCCGGACAG  960
ATAACCGAGG CTTCCTCTCC TTACAGTACA TGATTAGAAA TGAAGATGGG CAGATATGTT 1020
TTGTGGAATA TTACTGCTGC CCTGATGAAG AAGTTCCTGA GTCTTGAATA ATTCACTGAC 1080
TATTTCTGTC TTTTTTTTTT TTCTTTCACG AGACAGGGTT TCTCTGTAGC CCTGGCTGTC 1140
CTGGAACTCA CTTTGTAGAC CAGGCTGGCC TCGAACTCAG AAATTCACCT GCCTCTGCCT 1200
CCCGAGTGCT GGGATTAAAG GCATGCGCCA CCACGACCGG CGAATATTTG TGTCTTTATA 1260
GTTGGCGTTC TCACTCTAAC TCTCTAACCT TGATGATTTC ATTTTGGATT GTATAGAGAG 1320
ATGCATGGAG AAAATAGGAG CAAGGTCAGT CCCATTCTGT AAATCTGTAA ATAGTTTTGC 1380
GGCCGCGAAT TC                                                     1392
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Figure 1. Nucleotide sequences of *HRAD1* and *MRAD1*. Nucleotide sequences of the *HRAD1* (**A**) and *MRAD1* (**B**) cDNAs. The initiating and terminating codons are shown in bold. Consensus polyadenylation signals are underlined. Numbers to the right indicate the number of the last nucleotide on each line.

HRAD1 partially rescues the G_2 DNA damage checkpoint defects of *rad1* yeast mutants

The *HRAD1* ORF was subcloned into the *S.pombe* expression vector pART1 under control of the strong, constitutive *adh1*$^+$ promoter. Expression of *HRAD1* in a *rad1::ura4*$^+$ strain background increased the survival of these mutants following UV irradiation, to levels above that of the vector transformed control (Fig. 3A). However, this increase in viability did not reach the level of rescue that was obtained by expression of the wild type *rad1*$^+$ gene (Fig. 3A). Expression of *HRAD1* also restored partial resistance to ionizing radiation (Fig. 3B).

In order to more rigorously examine if *HRAD1* rescues the checkpoint defects of *rad1* mutants, *HRAD1* was expressed in a *rad1-1* strain containing the temperature sensitive *cdc25-22* allele. At the restrictive temperature of 36°C, these yeast arrest at the G_2/M transition point, due to their inability to activate the Cdc2 kinase. If cells blocked at the G_2/M transition are irradiated just prior to being released to the permissive temperature of 25°C, checkpoint proficient cells will undergo a dose dependent delay in entry into mitosis. Checkpoint deficient cells will enter mitosis without a noticeable delay. As shown in Figure 4A, the checkpoint deficient vector transformed controls enter a synchronous mitosis within 100 min of being irradiated, regardless of the dose received. Checkpoint proficient yeast expressing Rad1p undergo the characteristic dose dependent delay in entry into mitosis (Fig. 4B). Yeast expressing HRAD1p also undergo a dose dependent delay in entry into mitosis (Fig. 4C). The dose dependence is not equal to that of cells expressing Rad1p, however, this is what one would expect for partial rescue.

Expression of *HRAD1* restores minimal resistance to HU in *rad1::ura4*$^+$ yeast

Expression of *HRAD1* in Sp337 confers weak, but statistically significant resistance to the transient DNA synthesis inhibitor HU. However, this rescue is not nearly as high as that observed in other instances, such as *HRAD9* rescue of *rad9 S.pombe* mutants (27). As shown in Figure 5, *HRAD1* expressing cells lose viability with kinetics similar to that of the vector transformed control. Cells expressing wild type *rad1*$^+$ remain viable for at least 6 h in HU (Fig. 5).

DISCUSSION

We have identified novel human and mouse genes that are structural homologues of the fission yeast *rad1*$^+$ checkpoint control gene. The sequence similarity extends over the entire

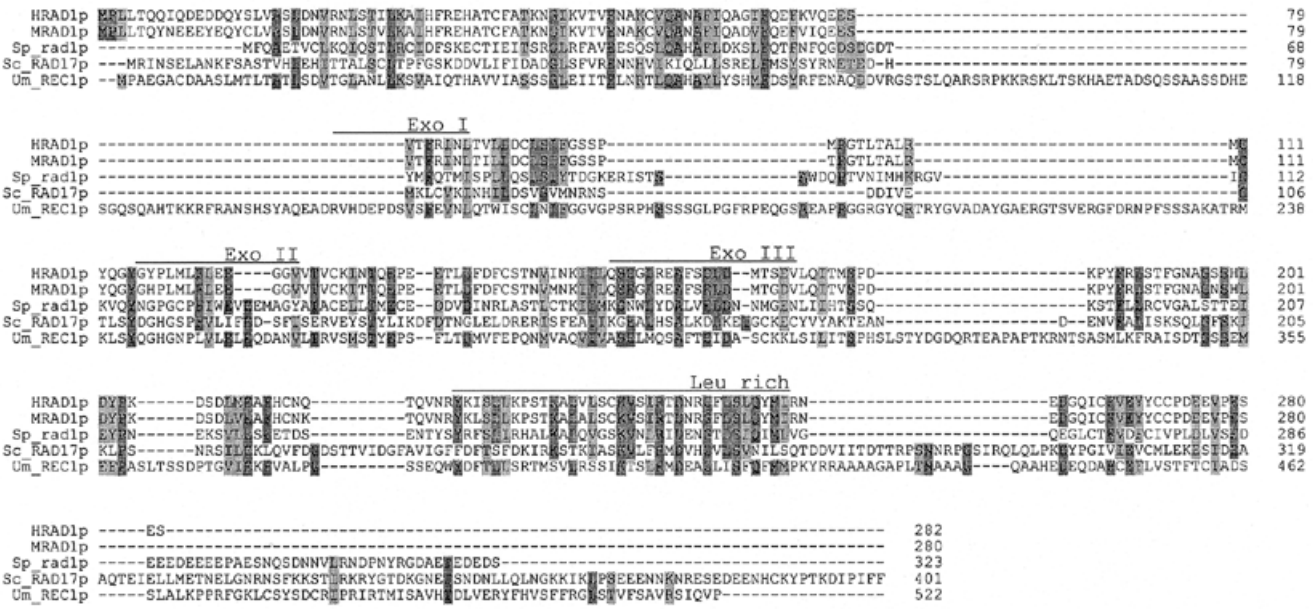

Figure 2. Amino acid sequence alignment of HRAD1p and MRAD1p with members of the Rad1p family. Amino acid alignment of HRAD1p, MRAD1p, *S.pombe* Rad1p (Sp_rad1p), *S.cerevisiae* RAD17p (Sc_RAD17p) and *U.maydis* REC1p (Um_REC1p). Numbers to the right indicate the numbering of the final amino acid on each line. Identical residues in ≥80% of the sequences are highlighted in dark grey. Conserved residues (defined in Materials and Methods) in ≥80% of the sequences are highlighted in light grey. Potentially important functional regions include the putative 3′→5′ exonuclease domains (Exo I, II and III), and the leucine rich region (Leu rich), which have been previously defined for other members of the family (35,36).

coding regions, indicating that the isolated cDNAs are full length. Particularly high levels of conservation were seen in two of three putative exonuclease domains, as well as in the leucine rich region that have been previously defined (35). The extent of amino acid conservation between HRAD1p and Rad1p, 27% identity and 53% similarity, is comparable to that observed between Rad1p and RAD17p (23% identity, 50% similarity). Rad1p and RAD17p have been shown by independent means to be involved in checkpoint control in fission and budding yeast, respectively (44). In different regions, HRAD1p and MRAD1p appear more like each of Rad1p, RAD17p and REC1p. Together with the functional complementation of *rad1* mutants by *HRAD1*, and the extent and pattern of structural similarity within this family, *HRAD1* and *MRAD1* are highly likely to be involved in mammalian G_2 checkpoint regulation.

While it has been clearly demonstrated that REC1p is a 3′→5′ exonuclease, it has also been demonstrated that this function is not required for checkpoint control by this protein (35). The sequence similarity between HRAD1p, MRAD1p and other members of the family over the exo II and exo III domains is high, but less so in the exo I domain. The role of the putative 3′→5′ exonuclease in HRAD1p function is questionable at this point.

We were able to show that *HRAD1* can partially rescue radiation sensitivity in *rad1* mutant yeast. This rescue is due to partial restoration of the G_2 checkpoint defect of these mutants, which is shown by the radiation-dose dependent delay experiment (Fig. 4). Checkpoint deficient vector transformed yeast begin to transit mitosis within 40 min of being released to the permissive temperature, regardless of the dose received. The checkpoint proficient yeast overexpressing Rad1p undergo a dose dependent delay in entry into mitosis. The unirradiated cells do not begin to transit mitosis until 60 min after release to the permissive temperature, which is 20 min later than the vector transformed cells. This difference is due to the additive effect of two cell cycle delaying influences, the overexpression of Rad1p and the *cdc25-22* mutation, which is not completely wild type even at the permissive temperature. Neither overexpression of Rad1p nor the *cdc25-22* allele alone is sufficient to cause the observed delay. Yeast *rad1* mutants overexpressing HRAD1p also undergo a dose dependent delay in entry into mitosis. The observed delay is not equivalent to that of the Rad1p expressing cells, but this is what one would expect for partial rescue. The maximal percentage of cells passing mitosis in both Rad1p and HRAD1p expressing yeast is lower than yeast carrying empty vector. This is due to the quality of the synchrony of cells passing mitosis. As the delay increases, the synchrony of the cells begins to diminish. Therefore, the highest percentage of cells passing mitosis is observed in the checkpoint deficient cells, where release from the block is quick. Checkpoint proficient cells will gradually lose synchrony over time and the maximal percentage of cells passing mitosis is lower.

This partial complementation suggests that *HRAD1* is the human homologue of fission yeast *rad1*$^+$. Cross species complementation by checkpoint genes has been demonstrated in other cases, but full complementation of all the defects of any particular mutant has not been observed. *HRAD9*, the human homologue of *S.pombe rad9*$^+$, restores resistance to HU in *rad9* null mutants, but fails to rescue UV sensitivity (27). *FRP1/ATR* is the human homologue of *S.cerevisiae MEC1/ESR1* and *S.pombe rad3*$^+$ (28,29,45). While *FRP1/ATR* will rescue some of the checkpoint defects of *MEC1/ESR1* mutants, it will not restore checkpoint proficiency to *rad3* mutants (29). Further analysis of *HRAD1* will be necessary to clearly define its role in human cell cycle checkpoint control.

In mammalian cells, the G_1 checkpoint is regulated in part by the *p53* and *ATM* genes, and defects in these genes have been associated with a variety of human cancers (3,4,8–11,16, 18,19,21,46,47). By contrast, very little is known about the

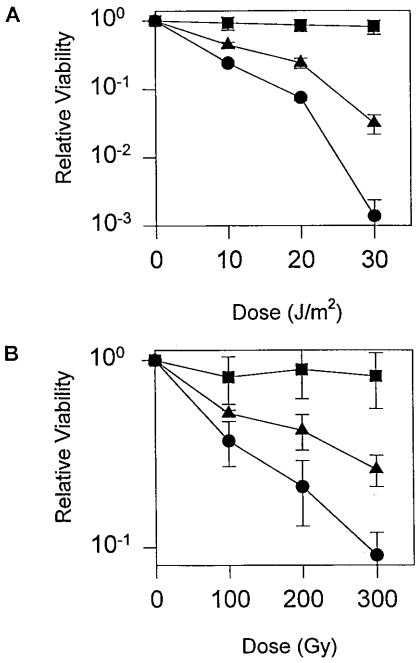

Figure 3. *HRAD1* expression restores resistance to DNA damage in *rad1::ura4*$^+$ mutants of *S.pombe*. Sp337 was grown to mid-logarithmic phase in PM media, plated on PM plates, and irradiated with the indicated doses of radiation. Colonies were counted after 6 days and relative viability is expressed as the number of irradiated cells relative to unirradiated cells that were able to form colonies. (**A**) The UV dose versus survival curve for Sp337 carrying pART1 (●), pART1-rad1$^+$ (■) or pART1-HRAD1 (▲). (**B**) The ionizing radiation dose versus survival curve. The symbols are the same as in (A). Both panels are the average of two independent experiments, each performed in duplicate. Error bars indicate standard error of the mean.

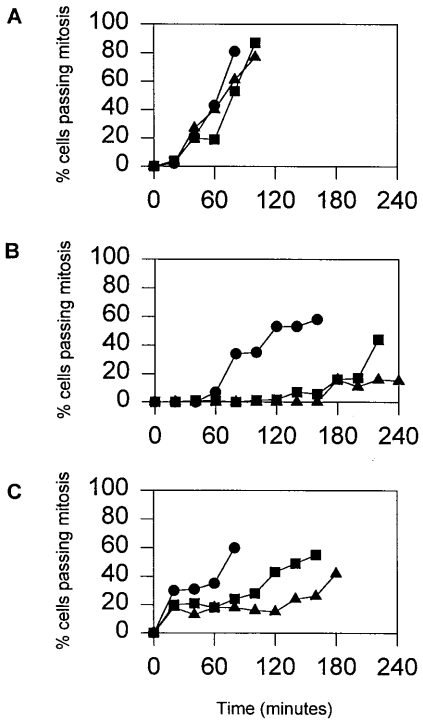

Figure 4. *HRAD1* expression restores dose dependent radiation-induced cell cycle delay to *rad1-1* mutants of *S.pombe*. Sp199 was grown to mid-logarithmic phase at 25°C, synchronized at the G_2/M transition by a 3 h incubation at 36°C, irradiated with the indicated doses of radiation (time zero), and released back to 25°C. At the indicated time points after irradiation, cells were removed, fixed, stained with DAPI and viewed under the fluorescence microscope. The % cells passing mitosis for each sample is the number of binucleate cells expressed as a percentage of the total number observed. Greater than 100 cells were scored for each timepoint. (**A**–**C**) Sp199 carrying either pART1 (A), pART1-rad1$^+$ (B) or pART1-HRAD1 (C). In each panel the doses were 0 J/m^2 (●), 10 J/m^2 (■) and 30 J/m^2 (▲). This figure is a representative example of three independent experiments.

molecular control of the G_2 checkpoint in mammalian cells. Like yeast, mammalian cells will respond to DNA damage or incompletely replicated DNA by arresting the cell cycle in G_2, prior to entry into mitosis. The presence of such a G_2 checkpoint has been shown to correlate with viability after exposure to radiation (48–52).

There are now three candidates for human G_2 checkpoint control genes: *HRAD1*, *HRAD9* and *FRP1/ATR*, homologues of the *rad1*$^+$, *rad9*$^+$ and *rad3*$^+$ genes of *S.pombe*. To date, none of these has been shown to function in human G_2 checkpoint control, though *HRAD1*, *HRAD9* and *FRPI/ATR* have been shown to rescue some of the defects in checkpoint deficient fission or budding yeast. Interestingly, BRCA1p co-localizes with the repair protein RAD51p, and both are found in regions of meiotic chromosomes similar to where FRP1p/ATRp is located (53,54). This spatial association with RAD51p and FRP1p/ATRp, and evidence that developmental arrest in *Brca1* null mice is partially rescued by a *p53* mutation indicates a role for BRCA1p in DNA damage repair (55). Genetic evidence from yeast indicates that *rad1*$^+$, *rad3*$^+$ and *rad9*$^+$ are part of the same G_2 checkpoint control pathway, and may form a physical complex. This suggests that HRAD1p, as the homologue of Rad1p, may be part of a multisubunit complex that includes other checkpoint proteins including HRAD9p, FRP1p/ATRp, RAD51p and BRCA1p.

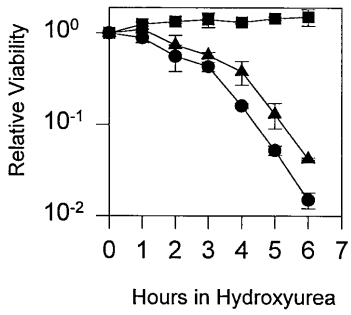

Figure 5. *HRAD1* expression does not restore resistance to transient DNA synthesis inhibition in *rad1::ura4*$^+$ mutants of *S.pombe*. Sp337 was cultured to mid-logarithmic phase at 32°C, HU was added to 12 mM (time zero), and aliquots of cells were removed at the indicated times and plated on PM media. Relative viability is expressed as the number of drug-treated versus untreated cells that were able to form colonies. The symbols represent Sp337 carrying either pART1 (●), pART1-rad1$^+$ (■) or pART1-HRAD1 (▲). The experiment was performed in duplicate and the error bars represent standard error of the mean.

It has been shown that caffeine treatment partially restores sensitivity to radiation in cell lines which have lost G_1 checkpoint control through the loss of p53 (56,57). Presumably, the loss of the ability to undergo apoptosis in response to radiation in p53 mutant cells leads to radiation resistance. Caffeine is presumed to eliminate the G_2 checkpoint in these cells, leading to radiation-induced death by premature mitosis, typical of checkpoint defective cells. Directly targeting *HRAD1* or HRAD1p could be an efficient way of targeting human G_2 checkpoint control. If elimination of G_2 checkpoint function would restore sensitivity to radiation or chemotherapeutic drugs to cells which have lost G_1 checkpoint function (i.e. $p53^{-/-}$ cells), there will be therapeutic benefits to inhibiting *HRAD1* or other G_2 checkpoint control genes and protein functions, in conjunction with radio- or chemotherapies.

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